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Synthetic and crystallographic insight into exploiting sp^2-hybridization in the development of α-L-fucosidase inhibitors

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Abstract: The sugar fucose plays a myriad of roles in biological recognition. Enzymes hydrolyzing fucose from glycoconjugates, α-L-fucosidases, are important targets for inhibitor and probe development. Here we describe the synthesis and evaluation of novel α-L-fucosidase inhibitors, with X-ray crystallographic analysis using an α-L-fucosidase from *Bacteroides thetaiotamicron* helping to lay a foundation for future development of inhibitors for this important enzyme class.

Many of the glycoconjugates that are found in eukaryotes bear terminal α-L-fucose residues; these glycans facilitate a variety of biological functions including cell-cell interactions, adhesion, inflammation and antigen determination.[1] Reflecting the important roles of this carbohydrate motif, there are specific enzymes, termed α-L-fucosidases (which are part of a larger class of enzymes termed glycoside hydrolases[2]), that act to cleave α-L-fucose moieties from glycoconjugates. This enzymatic process mediates the distribution of fucosylated glycans, and dysfunction or abnormal distribution of α-L-fucosidases in humans is associated with several disease states.[3-5]

As with other glycoside hydrolases, α-L-fucosidases have been classified into families based upon amino acid sequence homologies, with known α-L-fucosidases falling into GH families GH29 and GH95. Due to the importance of fucosidases and glycoside hydrolases in general, efforts have been made to understand the function of these enzymes focusing on substrate recognition and catalytic mechanism. This understanding has greatly benefited from the use of chemically synthesized small molecule inhibitors in concert with X-ray crystallography. Specifically for GH29 α-L-fucosidases, these enzymes retain the configuration, relative to the substrate, at the anomeric carbon after hydrolysis with the catalytic mechanism being demonstrated to proceed through a double-displacement mechanism with a covalent β-glycosyl-enzyme intermediate (Figure 1A).[6] Using X-ray crystallography, structures have been obtained of the glycosyl-enzyme intermediate[7] which have revealed a putative conformational itinerary for these enzymes of $^1C_4$-$^3H_4$-$^3S_1$ with the putative oxocarbenium ion-like

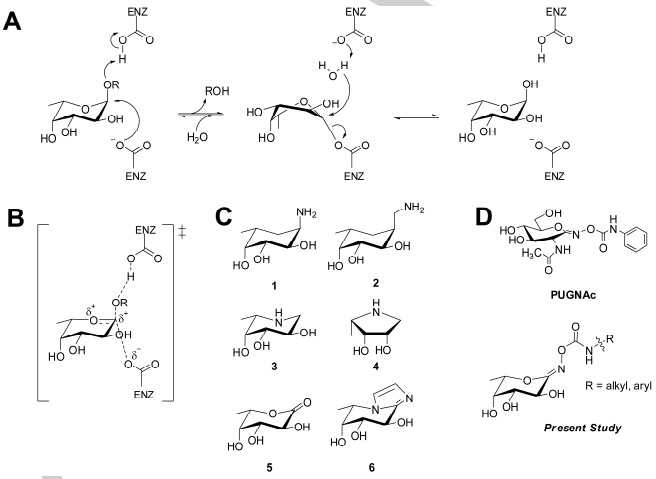

Figure 1. (A) GH29 fucosidases use a retaining catalytic mechanism involving a glycosyl-enzyme intermediate such that the overall reaction proceeds with net retention of stereochemistry. (B) Putative transition state for GH29 fucosidases. (C) Structures of known inhibitors of GH29 α-L-fucosidases. (D) Structure of the inhibitor PUGNAc and overall general structure of the corresponding inhibitors relevant to the described work described.

transition state around the $^3H_4$ conformation (Figure 1B).

In regards to inhibition, different aspects of the putative transition state, namely charge and shape, have been exploited giving compounds that act as inhibitors. For example compounds that mimic the charge of the putative transition state such as amines **1**[8] (and *N*-alkyl analogues[9]) and **2**[8] have been shown to be potent inhibitors of α-L-fucosidases (Figure 1C). Interestingly the 'C1 epimer' of **2** also displays good potency despite the molecule not adopting an α-L-fucose structure.[8] The deoxy analogue **3**[10] and pyrrolidine **4**[11] are also good inhibitors of these enzymes and compounds prepared that mimic shape such as **5**[12] and compounds such as **6**,[13] developed by Vasella, which are though to mimic the charge and shape of the transition state also display good potency.

In developing new types of inhibitors for GH29 α-L-fucosidases we were drawn to the inhibitor PUGNAc[14] (Figure 1D) which has been shown to be a highly potent inhibitor of retaining β-*N*-acetylhexosaminidases.[15-19] PUGNAc's potency is thought to lie in the compound possessing a transition state-like sp^2-hybridised carbon at C1, rendering it a potent inhibitor for β-*N*-acetylhexosminidase-catalysed reactions.[20, 21] In addition the oxime substituent is thought to provide additional binding energy by mimicking the aglycon of a natural substrate.[15]

With these points in mind we felt, based on the putative conformational itinerary analysis that has been described for GH29 α-L-fucosidases, that molecules bearing an sp^2-hybridized anomeric carbon may act as inhibitors of these enzymes as they could potentially mimic the conformation of the putative transition state (Figure 1D). In addition the inhibitory potency of

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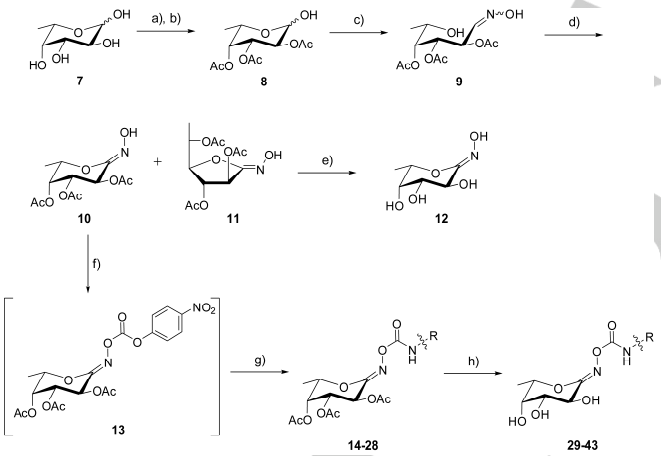
these molecules could potentially be varied by modifying the oxime substituent, as has been demonstrated in the development of PUGNAc-based analogues.[22]

Starting from L-fucose **7**, peracetylation under standard conditions followed by selective deacetylation at the anomeric position gave the known hemiacetal **8** (Scheme 1).[23] Treatment of **8** with hydroxylamine hydrochloride and pyridine gave the presumed oximes **9** which were carried through without further purification. Treatment of **9** with DBU and NCS allowed for oxidative ring closure but upon purification both the desired oxime **10** and the 1,4-hydroximolactone isomer **11** were isolated. The formation of analogous side products to the undesired material **11** have been observed in the preparation of other 1,5-hydroximolactones of this type.[24,25] Variation in temperature as well as the rate of addition of DBU did not improve the yield or selectivity for **10** but the yield was acceptable, so work continued on the functionalization of the hydroxyl motif. Before undertaking the preparation of various carbamates we first deprotected **10** using sodium methoxide in methanol which provided the 1,5-hydroximolactone **12**.

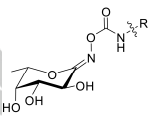
To access the panel of carbamates desired, we decided to utilize a method that has shown promise in the preparation of PUGNAc-based analogues where a suitable colourigenic carbonate is used as an intermediate as the formation of the desired carbamate can be indirectly monitored by observation of the liberated 4-nitrophenolate anion.[22] Thus, treatment of **10** with 4-nitrophenyl chloroformate gave the presumed carbonate **13**. Treatment of this carbonate **13** with various anilines and amines led to the formation of the carbamates **14**-**28**. The carbamates **14**-**28** were then deprotected to give the triols **29**-**43** in good yield.



Scheme 1. a) Ac_2O, pyr.; b) $EtNH_2$, AcOH, THF; c) $NH_2OH.HCl$, pyr., MeOH; d) DBU, NCS, CH_2Cl_2; e) NaOMe, MeOH; f) 4-nitrophenyl chloroformate, DIPEA, THF; g) Amine or aniline-based compound, DIPEA; h) NH_3, MeOH.

Next, to gain insight into the inhibitory potency of **12** and **29**-**43** against GH29 α-L-fucosidases we evaluated them against the α-L-fucosidase from bovine kidney (bFUCA1; one of the two α-L-fucosidases known to be expressed by *Bos Taurus*) using 4-nitrophenyl α-L-fucopyranoside as a substrate. All the compounds displayed activity as inhibitors against bFUCA1 (Table 1). The 1,5-hydroximolactone **12** was a weak inhibitor (323 ± 12 μM) of the enzyme, which demonstrates the potential importance of the phenyl carbamoyl motif. This is in accordance with what has been observed for other inhibitors using this scaffold to target other enzyme classes[15,18] and also for other inhibitor scaffolds trying to mimic the transition state of GH29 α-L-fucosidase-mediated reactions.[26] Some trends were apparent in the relative activities of the carbamates. The *N*-alkyl chain carbamates **29**-**32** increased in potency with increasing chain length. This correlates with what has been observed for some carbasugar inhibitors of GH29 α-L-fucosidases[9,27] and may indicate fortuitous hydrophobic binding interactions in or around the active site of the enzyme. Conversely, the activities of the other *N*-alkyl carbamates **33** and **34** decreased with increasing steric bulk close to the carbamoyl functional group. The relative activities of the cyclic alkyl carbamates **35**-**38** were in agreement with this observation; those with larger pendant rings were weaker inhibitors of the enzyme with this loss of activity most likely arising due to steric and electronic effects. Interestingly, significant increases in potency were observed for the *N*-aryl carbamates compared to the parent hydroximolactone **12**.

Table 1. Inhibition constants of prepared inhibitors against bovine kidney α-L-fucosidase bFUCA1.



29-43

Inhibitor	R	K_i (μM)	Inhibitor	R	K_i (μM)
29	$(CH_2)_2CH_3$	190±11	**37**	$CH(CH_2)_4$	325±8
30	CH_2CHCH_2	175±6	**38**	$CH(CH_2)_5$	580±7
31	$(CH_2)_3CH_3$	172±7	**39**	Ph	11±1
32	$(CH_2)_5CH_3$	143±8	**40**	*p*-BrPh	85±4
33	$CH(CH_3)_2$	320±12	**41**	*p*-MeOPh	9.5±0.9
34	$C(CH_3)_3$	440±9	**42**	$4\text{-}CH_3(CH_2)_3Ph$	7.5±0.4
35	$CH(CH_2)_2$	184±6	**43**	$4\text{-}CH_3(CH_2)_9Ph$	4.8±0.5
36	$CH(CH_2)_3$	220±12			

Comparison of **39** with the *N*-cyclohexyl carbamate **38** demonstrates that given the similar shape, size, and hydrophobicity of the pendant moieties in these two inhibitors, the drastic increase in activity seems to be associated with the aromaticity of the pendant moiety. As such, it is likely that either specific interactions with the aromatic ring, such as π-stacking, or general hydrophobic effects and desolvation contribute to the strong activity of the inhibitors bearing pendant *N*-aryl carbamates. In addition, due to the increased potency of **41**-**43** further evidence is provided for a putative hydrophobic binding pocket within the active site of bFUCA1, which may be exploited for the development of improved inhibitors.

In the absence of a credible mammalian structural candidate, in order to gain a more detailed understanding of the molecular basis for the inhibition of GH29 α-L-fucosidases, we obtained a 1.7 Å crystal structure of **39** in complex with the α-L-fucosidase BT2970 from *Bacteroides thetaiotamicron* (See Supporting Table). BT2970 shares 27% identity with human FUCA1 and 27% identity with bFUCA1, including near complete identity of active site residues (See Supporting Figure).

After soaking crystals of BT2970 with **39** (K_i of **39** against BT2970 equals 121 ± 24 μM), clear electron density corresponding to the ligand was observed in the active site of

BT2970 *in crystallo* (PDB code: 6HZY). As expected from the conformational itinerary, C3 lies "above" the ring plane with **39** adopting a ^{3}E conformation. In our experiments, BT2970 crystallized in the I2 space group, with two molecules of protein in the asymmetric unit; both copies of BT2970 contained active site density for **39**. Whilst electron density for the fucose portion of **39** was clear, the density grew progressively weaker along the 'aglycon' portion of the ligand, likely reflecting increased molecular disorder as the ligand exits the enzyme active site. Although counter-intuitive, PUGNAc and related inhibitors with *N*-aryl carbamates are normally tighter-binding compounds than the parent hydroximolactones, but frequently the *N*-aryl group is

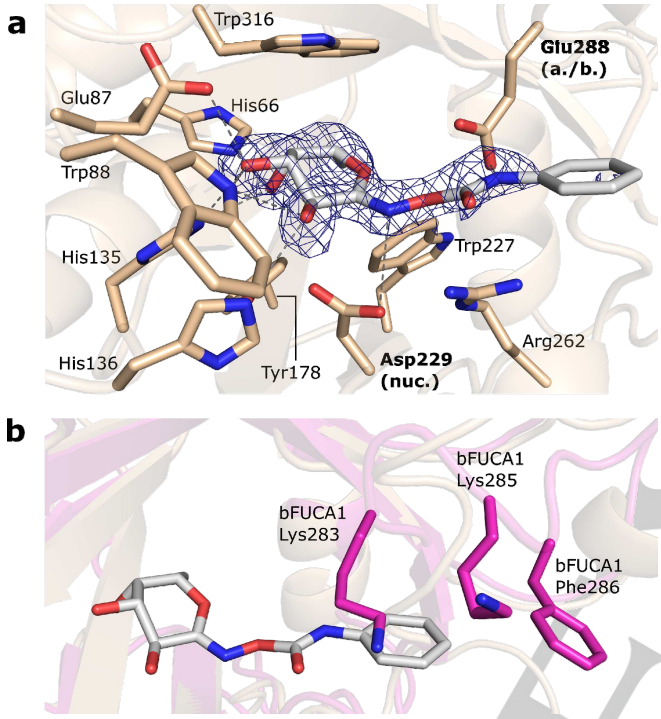


Figure 2. a) 'Side' view of **39** bound in the active site of BT2970, showing direct H-bonding interactions to nearby residues. The ligand shown is from chain B in the BT2970 crystal structure. Electron density is REFMAC maximum-likelihood/σ_A weighted $2F_o-F_c$ contoured to 0.29 electrons/Å^3. b) Superposition of a bFUCA1 homology model (purple) against the ligand position from the BT2970-**39** complex, showing Lys283, Lys285 and Phe286 of the bFUCA1 homology model near the phenyl ring of **39**. For clarity, electron density and BT2970 sidechains have been omitted from this image.

disordered in crystal (as for example seen in *Vc*NagZ[18,20]). The inhibitor **39** was observed to make similar H-bonding interactions within the BT2970 active site as previously observed for complexes with other fucose configured ligands[7], albeit the bulky 'aglycon' of **39** induced a degree of displacement of the catalytic acid/base residue Glu288, leading to disorder of the loop containing this residue.

Due to the lack of interactions observed between BT2970 and the phenyl ring of **39**, it was not possible to analyze any enthalpic contributions of this moiety to binding in BT2970. In order to predict the possible interactions **39** might make with mammalian fucosidases, we generated a RaptorX homology model of the bovine α-L-fucosidase bFUCA1, templated upon the structure of a fucosidase from *Thermatoga maritima* (TM0306; PDB accession 2ZWY).

Superposition of the BT2970-**39** ligand complex with the bFUCA1 RaptorX model suggested that Lys283, Lys285 and Phe286 of bFUCA1 would reside in a loop near the projected phenyl moiety of bound **39**, raising the possibility of cation-π or π-π interactions between bFUCA1 and the phenyl ring of **39**, which are not present in BT2970 (Figure 2B). Lys285 and Phe286 are fully conserved between bovine and human FUCA1, and replaced by closely related Arg and Tyr residues in the closely related bovine and human FUCA2 enzymes (See Supporting Figure). The additional cation-π or π-π interactions available to mammalian GH29 enzymes may produce more potent inhibition for ligands with aromatic 'aglycons' compared to those with similarly sized aliphatic 'aglycons'.

In conclusion, we have developed new tools to study GH29 α-L-fucosidases. The phenyl carbamate **39** and the aryl derivatives **41**-**43** are potent inhibitors of GH29 α-L-fucosidases, with the sp^2-hybridized centre contributing to potency. The benefit of alkyl and aryl substituents does not simply reflect improved interactions, since - as observed in other systems - the aryl groups are disordered, but may also come from solvent reorganization upon binding. Collectively, the results obtained here suggest that inhibitors of this type, that mimic the shape of the ring at the transition state, have a place in the development of inhibitors of α-L-fucosidases.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: enzyme inhibitors • fucosidases • glycosidases • hydroximolactone • carbamate

[1] M. Schneider, E. Al-Shareffi, R. S. Haltiwanger, *Glycobiology* **2017**, *27*, 601.
[2] B. L. Cantarel, P. M. Coutinho, C. Rancurel, T. Bernard, V. Lombard, B. Henrissat, *Nucleic Acids Res.* **2009**, *37*, D233.
[3] E. Miyoshi, K. Moriwaki, N. Terao, C. C. Tan, M. Terao, T. Nakagawa, H. Matsumoto, S. Shinzaki, Y. Kamada, *Biomolecules* **2012**, *2*, 34.
[4] E. Maverakis, K. Kim, M. Shimoda, M. E. Gershwin, F. Patel, R. Wilken, S. Raychaudhuri, L. R. Ruhaak, C. B. Lebrilla, *J. Autoimmun.* **2015**, *57*, 1.
[5] A. Varki, *Glycobiology* **2017**, *27*, 3.
[6] F. A. Shaikh, A. Lammerts van Bueren, G. J. Davies, S. G. Withers, *Biochemistry* **2013**, *52*, 5857.
[7] A. Lammerts van Bueren, A. Ardèvol, J. Fayers-Kerr, B. Luo, Y. Zhang, M. Sollogoub, Y. Bleriot, C. Rovira, G. J. Davies, *J. Am. Chem. Soc.* **2010**, *132*, 1804.

[8] S. Ogawa, A. Maruyama, T. Odagiri, H. Yuasa, H. Hashimoto, *Eur. J. Org. Chem.* **2001**, 967.
[9] S. Ogawa, M. Mori, G. Takeuchi, F. Doi, M. Watanabe, Y. Sakata, *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2811.
[10] G. W. J. Fleet, A. N. Shaw, S. V. Evans, L. E. Fellows, *J. Chem. Soc, Chem. Commun.* **1985**, *1985*, 841.
[11] C. H. Wong, L. Provencher, J. A. Porco, S. Jung, Y. Wang, L. Chen, R. Wang, D. H. Steensma, *J. Org. Chem.* **1995**, *60*, 1492.
[12] G. Y. Wiederschain, E. L. Rosenfeld, *Bull. Soc. Chim. Biol. (Paris)* **1969**, *51*, 1075.
[13] A. Peer, A. Vasella, *Helv. Chim. Acta* **1999**, *1044-1065*.
[14] D. Beer, J. L. Maloisel, D. M. Rast, A. Vasella, *Helv. Chim. Acta* **1990**, *73*, 1918.
[15] M. Horsch, L. Hoesch, A. Vasella, D. M. Rast, *Eur. J. Biochem.* **1991**, *197*, 815.
[16] M. S. Macauley, G. E. Whitworth, A. W. Debowski, D. Chin, D. J. Vocadlo, *J. Biol. Chem.* **2005**, *280*, 25313.
[17] E. Ficko-Blean, K. A. Stubbs, O. Nemirovsky, D. J. Vocadlo, A. B. Boraston, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 6560.
[18] K. A. Stubbs, M. Balcewich, B. L. Mark, D. J. Vocadlo, *J. Biol. Chem.* **2007**, *282*, 21382.
[19] T. Sumida, K. A. Stubbs, M. Ito, S. Yokoyama, *Org. Biomol. Chem.* **2012**, *10*, 2607.
[20] M. Balcewich, K. A. Stubbs, Y. He, T. James, G. J. Davies, D. J. Vocadlo, B. L. Mark, *Protein Sci.* **2009**, *18*, 1541.
[21] Y. He, M. S. Macauley, K. A. Stubbs, D. J. Vocadlo, G. J. Davies, *J. Am. Chem. Soc.* **2010**, *132*, 1807.
[22] M. Hattie, N. Cekic, A. W. Debowski, D. J. Vocadlo, K. A. Stubbs, *Org. Biomol. Chem.* **2016**, *14*, 3193.
[23] V. Dulery, O. Renaudet, C. Philouze, P. Dumy, *Carbohydr. Res.* **2007**, *342*, 894.
[24] H. Mohan, A. Vasella, *Helv. Chim. Acta* **2000**, *83*, 114.
[25] K. A. Stubbs, N. Zhang, D. J. Vocadlo, *Org. Biomol. Chem.* **2006**, *4*, 839.
[26] S. Ogawa, M. Watanabe, A. Maruyama, S. Hisamatsu, *Bioorg. Med. Chem. Lett.* **2002**, *12*, 749.
[27] H. Wu, C. Ho, T. Ko, S. D. Popat, C. Lin, A. H. Wang, *Angew. Chem. Int. Ed.* **2010**, *49*, 337.

Entry for the Table of Contents (Please choose one layout)

Layout 1:

COMMUNICATION

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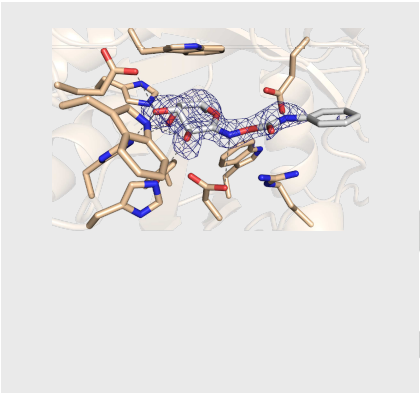

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